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ISOLATION OF MYCOLIC ACID-CONTAINING GLYCOLIPIDS IN *NOCARDIA RUBRA* AND THEIR GRANULOMA FORMING ACTIVITY IN MICE

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Three classes of glycolipids (TMM (trehalose monomycolate), TDM (trehalose dimycolate) and GM (glucose mycolate)) containing mycolic acids as hydrophobic components were isolated from a strain of *Nocardia rubra (Rhodococcus rubrum)* and their structures have been partially characterized using infrared spectrometry, gas-liquid chromatography and gas chromatography-mass spectrometry. Acid or alkaline hydrolysis of isolated glycolipids revealed that trehalose was the sole water soluble component in TMM and TDM, while glucose was the hydrophilic component in GM. On the other hand, saturated, monoenoic and dienoic mycolic acids with carbon atoms ranging from C_{36} to C_{50} contained constituents of fatty acid moiety at C_{44}. From the analytical results, TMM, TDM and GM were tentatively identified as trehalose monomycolate, trehalose dimycolate and glucose monomycolate, respectively. The mycolic acid composition differed significantly by the glycolipid classes: the highest amount of saturated mycolic acids were detected in TMM and GM, while a significant amount of dienoic mycolic acids have been found in TDM and the cell wall bound lipid fraction (BL).

All these three classes of glycolipids containing mycolic acids showed strong granuloma forming activity in lungs and spleen of ICR mice 1 week after intravenous injection of 100 to 500 μg glycolipid in W/O/W micelles containing Freund's incomplete adjuvant. These results indicated that glycolipids containing shorter carbon chain mycolic acids ranging C_{40-50}, corresponding to less acyl numbers or monosaccharides such as glucose, can also produce foreign body-type granuloma in mice without protein antigens.

Keywords —glycolipid; trehalose dimycolate; mycolic acid; acil-fast bacterium; granuloma formation

INTRODUCTION

The most characteristic component of cell walls of the bacteria belonging to Actinomycetales, such as Mycobacteria and Nocardia, has been recognized to be "mycolic acid", a very high molecular weight 3-hydroxy fatty acid with a long chain alkyl branch at the 2-position. These acids are known to be associated with cell wall skeleton *via* the esterification at the 5-position of arabinose or with specific glycolipids such as cord factor or trehalose monomycolate.[1)]

The mycolic acids play important roles for the maintenance of hydrophobic properties and acid-fastness of the cell walls and their composition varies, not only in genera or species, but also adaptatively in response to the environmental conditions, such as growth temperature.[2)] On the other hand, the cell wall components containing mycolic acids possess unique biological activities between the host and parasite relationships in infectious diseases, such as immunoadjuvant activities, macrophage activating activities and antitumor activities.[3)] It has been previously reported that BCG cell walls or *Nocardia rubra* cell walls cause a significant increase in lung weight when injected intravenously into mice.[4)] Histologically, it has been revealed that the increase in lung weight results from massive granuloma formation.[4i, 5)] Furthermore, Meyer *et al.* reported[4d, 6)] that a cord factor (P_3) from BCG, may play an important role in pulmonary granuloma formation by BCG CWS vaccination, probably by the co-operation of protein nature antigenic substances and adjuvant active glycoli-

pids.

However, owing to the highly hydrophobic properties, mycobacterial cord factors possess strong toxicity for mice and therefore, appear to be unsuitable as antitumor agents or adjuvant active compounds for human use. On the other hand, to date, the biological activities of isolated glycolipids from *Nocardia* and related taxa have not been demonstrated in detail. We have examined the composition of mycolic acid-containing glycolipids in *Nocardia, Rhodococcus* and *Gordona* which are closely related taxonomically to *Mycobacteria,* analyzed their structures and tested the granuloma forming activity in mice.

We report here three classes of glycolipids possessing mycolic acids as their hydrophobic moiety and trehalose or glucose as their hydrophilic moiety from *N. rubra* and related *Rhodococcus* group, which can produce massive granulomatous changes in the lungs and spleen of mice.

MATERIALS AND METHODS

Growth of the Organisms — *N. rubra* M-1 and other related bacteria were cultivated in a medium containing 1% glucose, 0.2% yeast extract and 0.5% peptone, with the pH adjusted to 7.0. Incubations were carried out in a rotary shaker with vigorous shaking for 5 d at 30 °C.

Separation and Analysis of Lipids — After the cells were harvested by centrifugation, the lipids were extracted twice with 20 vol of chloroform-methanol (2:1, by vol) from the cells and then, the lipids were separated by a thin-layer chromatography. For the separation of glycolipids containing mycolic acids, a mixed solvent of chloroform-methanol-acetone-acetic acid (90:10:6:1, by vol) (1) was used and for the separation of mycolic acid methyl esters, *n*-hexane-diethylether (4:1, by vol) (2) was used. The chromatograms were sprayed with 50% H_2SO_4 or dipped into iodine vapor and heated at 180 °C for 20 min (charring) to locate the lipids. The glycolipids were detected by heating the chromatograms after spraying with anthrone-H_2SO_4 reagent while phospholipids were detected by spraying the chromatograms with Dittmer's reagent.[7] The glycolipids were purified on a preparative thin-layer plate of silica gel (Uni plate) (Analtech, Delaware, U.S.A.) repeatedly until a single spot was obtained and then, the isolated glycolipids were analyzed. Infrared (IR) spectrometry was carried out with Nippon Bunko Apparatus. The component sugars were identified by gas chromatography (GC) (Hitachi 063, equipped with a 1.0 m × 3 mm glass coiled column with 5% SE-30) as their trimethylsilylether (TMS) derivatives of methyl glycosides after transmethylation with 3% anhydrous HCl for 14 h at 90 °C. The oligosaccharide structure was determined by a gas chromatographic and mass spectrometric analysis of TMS derivatives (2% OV 101 column) after a mild alkaline hydrolysis (0.2N KOH methanol-chloroform, 2:1, by vol) for 1 h at room temperature. Excess alkali was removed with Amberlite IR and the water removed *in vacuo.* The glycolipids were partially hydrolyzed with 0.05N KOH methanol-chloroform (2:1, by vol) for 5 to 40 min at room temperature and the products were analyzed by thin-layer chromatography (TLC) with the solvent system described above (1) and after neutralization with acetic acid.

Gas Chromatography-Mass Spectrometry (GC-MS) — The molecular species composition of mycolic acids was determined by a GC-MS system (Hitachi M-80B) as their trimethylsilylether derivatives of methyl esters as reported previously.[2,8] The gas chromatographic columns (0.2 m × 3 mm glass coiled) with 2% OV-101 were maintained at 300 °C for the separation of C_{30-50} mycolic acids. Both the separator and the injector were kept at 370 °C. The ionization energy was 20 eV and the accelerating voltage was 3.2 kV. The mass spectra of trimethylsilylated methyl mycolate of samples from the maximal points of the gas chromatographic peaks were recorded, while mass chromatograms were recorded by monitoring (M-15) ions, due to the loss of a methyl group from individual TMS methyl mycolate. The mycolic acid composition of individual glycolipid was calculated from peak area percentages.

Granuloma Formation by Glycolipids in Mice — For the examination of granuloma forming activity, each glycolipid suspended in phosphate buffered saline was added to an equal volume of Freund's incomplete adjuvant (FIA) from Difco in a teflon grinder and the mixture was ground to prepare a water-in-oil emulsion, as reported in previous papers.[16] Furthermore, the saline containing 0.2% Tween 80 was added to the water-in-oil and ground again, producing a water-in-oil-in-water emulsion containing a

final FIA concentration of 3%. Samples of 0.1 ml containing 0 to 500 μg of glycolipid were injected into the tail vein of ICR mice (male, 4—5 w age, about 20 g). One week after an intravenous injection of glycolipids, mice were anaesthesized with diethylether and sacrificed. The lungs and spleens were immediately removed by dissection and the wet weight of the pulmonary and spleenic granulomas were measured. The contamination of the blood cells was negligible. For controls, mice injected with water in oil in water emulsion without glycolipids were killed and their organs weighted. The lung or spleen index was calculated as follows:

$$\text{lung (or spleen) index} = \frac{\text{lung (or spleen) weight}}{\text{body weight}} \times 100$$

For a more detailed evaluation of cellular infiltration in granulomatous tissues, the lungs and spleen were prepared for histological examination. They were fixed in 10% Formalin and stained with hematoxylin and eosin. The morphological observations were fully carried out with a light or an electron microscope.

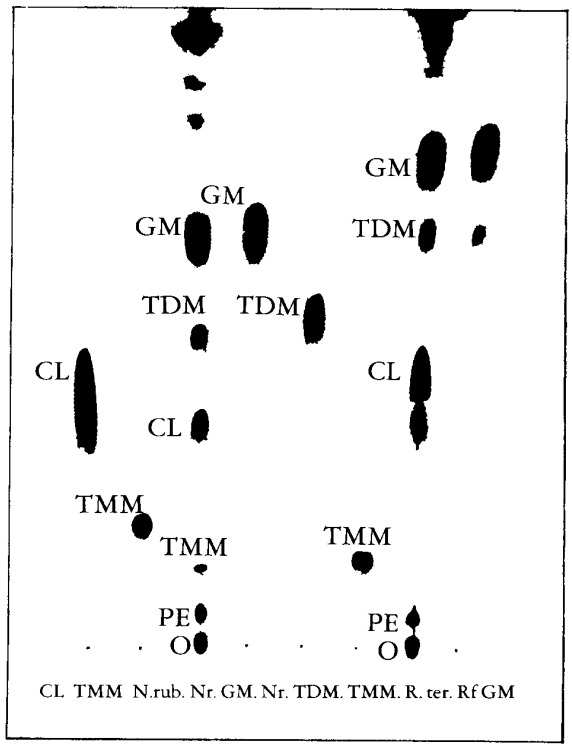


FIG. 1. *Thin-Layer Chromatograms of the Extractable Lipids from N. rubra and Related Taxa*
1) The total extractable lipids from N. rubra; 2) isolated "GM"; 3) isolated TDM; 4) isolated TMM: 5) the total extractable lipids from Rhodococcus terrae 70012 and 6) isolated GM (upper spot) and TDM (lower spot). Plate was developed with the solvent (1) in the text. The glycolipids were detected with the anthron reagent spray and heating at 160 °C for 20 min.

RESULTS

Separation and Isolation of Mycolic Acid-Containing Glycolipids in N. rubra

The chloroform-methanol soluble lipids obtained from *N. rubra* accounted for about 2.5% of the packed cell weight. TLC of the extractable lipids from *N. rubra* with solvent system (1) showed that three classes of glycolipids designated as trehalose monomycolate (*Rf* 0.15), trehalose dimycolate (TDM) (*Rf* 0.50) and glucose mycolate (GM) (*Rf* 0.70), all showing anthron positive reaction and possessing mycolic acids as their hydrophobic components, existed (Fig. 1). The *Rf* values of TMM and TDM were close to those of trehalose mono- and dimycolate respectively, from *Mycobacterium tuberculosis* $H_{37}Rv$. However, it was noted that they were slightly lower than those of Mycobacterial cord factors. The total amount of glycolipids accounted for approximately 45—60 percent of the extractable lipids (depending on culture stages) and the most abundant glycolipid was GM, possessing the highest *Rf* value on the TLC (about 60 percent of the total glycolipids). The IR spectra of purified glycolipids showed a deep and wide absorption in the region of 3300—3500 cm^{-1} due to hydroxyl groups. Absorptions due to C-H stretching of CH_2 and CH_3 were observed between the 2800—2900 cm^{-1} region and a carboxyl ester band appeared at 1720—1740 cm^{-1}. The absorption band at 1460 cm^{-1} indicated $C\text{-}CH_2$ groups and the band at the finger print region between 990 cm^{-1} and 1200 cm^{-1} resembled those of mycobacterial cord factors (Fig. 2).

Structure Analysis of Hydrophilic Moiety of Glycolipids in N. rubra

After acid methanolysis of each glycolipid with 3% anhydrous HCl methanol, the aqueous phase was separated, evaporated *in vacuo* and then, trimethylsilylated methyl glycosides were analyzed by gas chromatography. All the TMS methyl glycosides obtained from each glycolipid yielded a peak(s) corresponding essentially to authentic TMS methyl glucoside. On the other hand, the aqueous phase from the glycolipids,

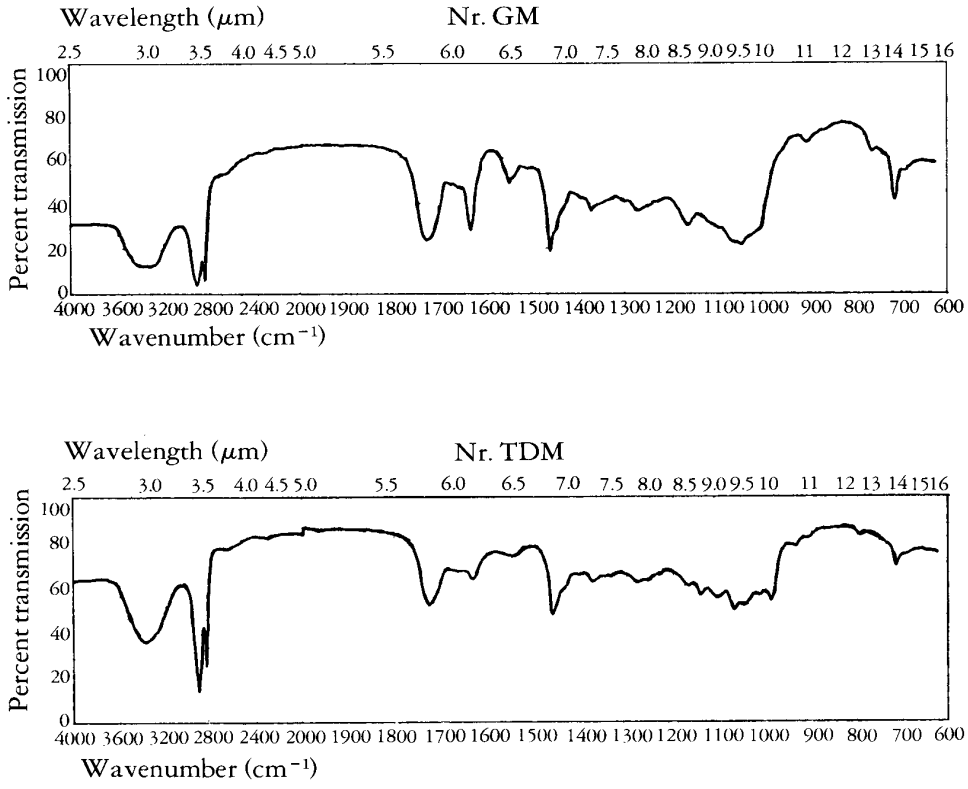


FIG. 2. *Infrared Spectra of Mycolic Acid-Containing Glycolipids Isolated from N. rubra*
IR spectra were recorded with KBr discs using Nippon Bunko Apparatus.

TMM and TDM, after alkaline hydrolysis revealed only one peak with a retention time of trehalose on GC, while that from the glycolipid GM produced one peak with a retention time of glucose (Fig. 3). For further structure analysis of glycolipids, a mild alkaline hydrolysis was performed and the products were developed by TLC. After a short time hydrolysis (5—60 min) of TDM with 0.5 N KOH methanol-chloroform, a glycolipid migrating essentially with TMM and free mycolic acid appeared on TLC, while no such intermediate glycolipid formation was observed after the hydrolysis of GM (Fig. 4). On the other hand, *n*-hexane extracts after the methanolysis of each glycolipid contained only methyl mycolate on TLC but not normal (nonpolar) fatty acid methyl esters in any detectable quantities. From these results, the above three glycolipids were tentatively identified as trehalose monomycolate for TMM, trehalose dimycolate for TDM and glucose monomycolate for GM.

Structure Analysis of Hydrophobic Moiety of Glycolipids in N. rubra

The mycolic acid composition of each glycoli-

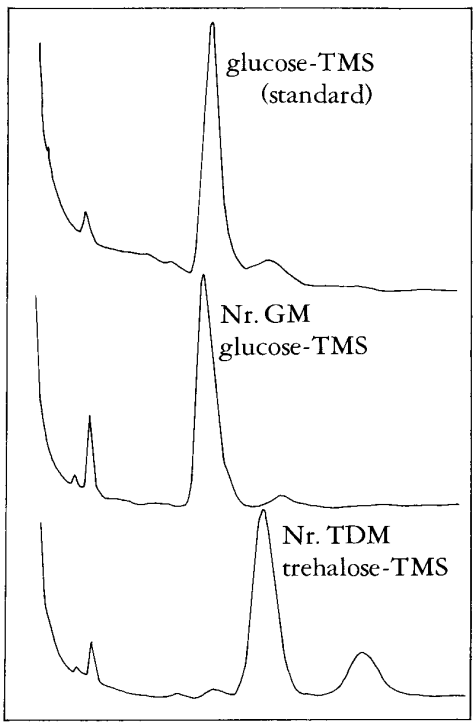


FIG. 3. *Gas Chromatograms of TMS Derivatives of Oligosaccharides Obtained after Alkaline Hydrolysis of Individual Glycolipid from N. rubra*
The conditions for gas chromatography are described in the text.

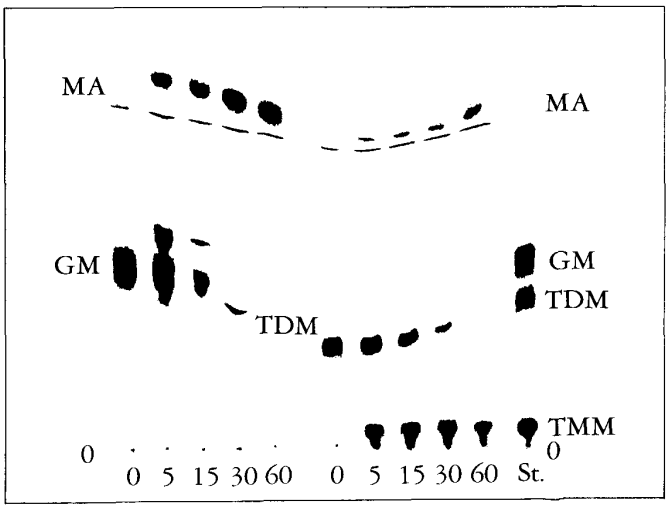


FIG. 4. *Thin-Layer Chromatograms of the Lipophilic Products Obtained after the Partial Alkaline Hydrolysis of Isolated Glycolipid from N. rubra*
Plate was developed with the solvent (1) in the text.

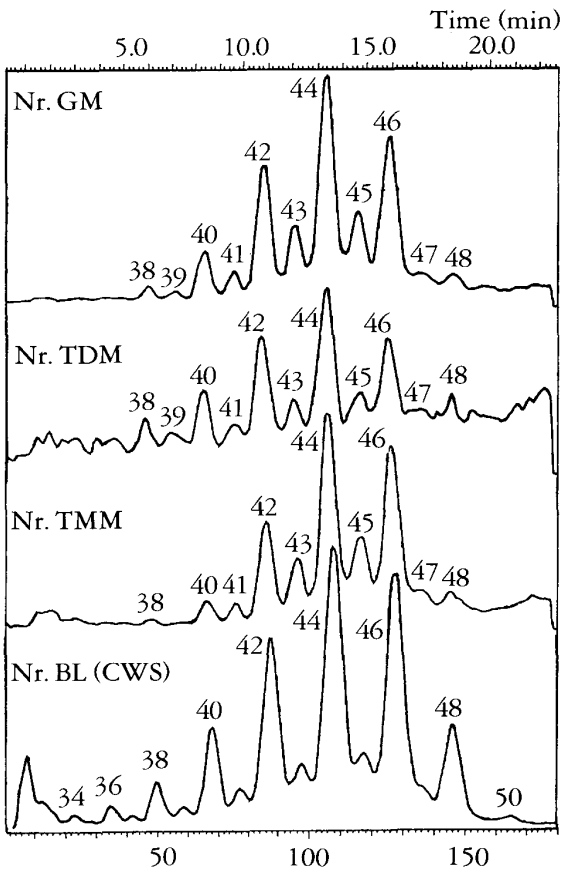


FIG. 5. *Gas Chromatograms of TMS Methyl Mycolate of the Individual Glycolipid Classes in N. rubra*
Gas chromatographic analysis was performed with a glass coiled column (3 mm i.d. × 0.5 m) with 2% OV-101 on chromosorb W at 300 °C. Other conditions are described in the text.

pid from *N. rubra* was fully examined by a GC-MS system. As shown in Fig. 5, the gas chromatographic analysis of TMS-methyl mycolates from each glycolipid (TMM, TDM, GM and BL-cell wall bound lipid) gave a clear separation according to the total number of carbon atoms. The gas chromatographic patterns of the mycolic acid molecular species of each glycolipid resembled each other, ranging from C_{36} to C_{50}, and the most abundant species was C_{44}. However, the concentrations of odd carbon numbered mycolic acids differed significantly according to the glycolipid classes.

Furthermore, when the mass spectra were recorded at the top of gas chromatographic peaks, the (M-15) ions due to the loss of methyl group from molecular ions of TMS methyl mycolate were duplicated or triplicated, indicating the specific carbon numbered mycolic acids consisted of more than two homologues differing in the numbers of double bond (Fig. 6). Fragment ion (A) due to C_{2-3} cleavage, indicating the carbon and double bond numbers of the straight chain alkyl unit of 30-TMS methyl mycolate, also gave douplet or triplet differing by two mass numbers unit, suggesting the one or two double bond(s) were located on the straight chain of mycolic acids. On the other hand, fragment ion (B) due to C_{3-4} cleavage, indicating the carbon and double bond numbers of α-branched chain alkyl unit gave a singlet at *m/z* 371, 343 or 315, suggesting branched chain at the 2-position were saturated C_{10}, C_{12}, or C_{14}in all the mycolic acid species in *N. rubra*. To fully determine the molecular species composition of mycolic acids from each glycolipid classes, mass chromatography was performed, monitoring (M-15) ions of saturated, monoenoic and dienoic derivatives of each carbon numbered species. Figure 7 shows mass chromatograms of the TMS methyl mycolates obtained from GM and BL (bound lipid fraction) and there were marked differences in the ratios of saturated, monoenoic and dienoic mycolic acids between both chromatograms.

In glucosyl mycolate, saturated mycolic acids were most abundant in all carbon numbered classes with small quantities of monoenoic acids,

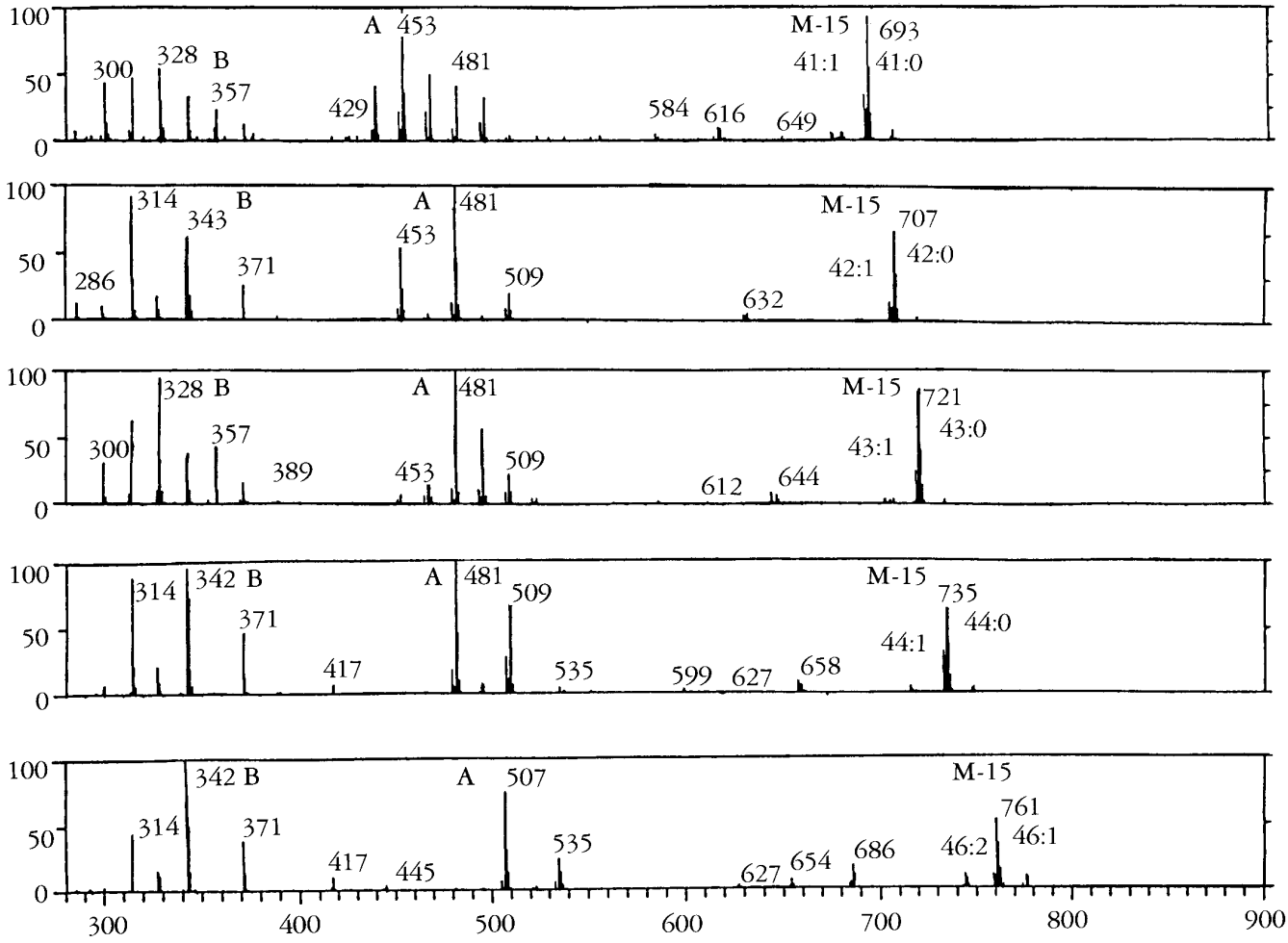


FIG. 6. *Mass Spectra of TMS Methyl Mycolates of the Glycolipid "GM" in N. rubra*
Mass spectra were recorded with Hitachi M-80B double focussing apparatus. The conditions for mass spectrometry are described in the text.

while in bound lipid fraction, mono- and dienoic mycolic acids occurred abundantly. The composition of each molecular species of mycolic acid was calculated from the peak areas and the results are summarized in Table I. It was noted that mycolic acid composition of all four glycolipid classes: TMM, TDM, GM and BL (cell wall bound) differed significantly; glucose mycolate contained mostly saturated mycolic acids, while the bound lipid fraction possessed more highly unsaturated species. Also, glucose mycolate and trehalose monomycolate possessed abundantly odd carbon numbered mycolic acids.

Granuloma Forming Activity by the Isolated Glycolipid in Mice

For the examination of granuloma formation, ten ICR male mice in a group were injected into the tail vein with the emulsion containing 10 to 500 μg of glycolipid (or unfractionated lipids) without protein antigen. One week after the injection of the heat-killed cells of *N. rubra* (0.1 to 3 mg) or the unfractionated lipids (100 to 500 μg) in W/O/W micelles, a marked increase in granuloma formation in the lungs or spleen index was observed (Fig. 8). Among the major cellular lipids of *N. rubra,* only glycolipids containing mycolic acid, TMM, TDM and GM showed activity for granuloma formation in lungs and spleen in mice, while phospholipids such as phosphatidylethanolamine and diphosphatidylglycerol did not show any activity (Fig. 9). The dose responses for granuloma formation by the isolated glycolipid "GM" and "TDM" are demonstrated in Fig. 10 (A) and (B). It was noted that the increase in lung index was significantly higher than that in spleen index up to 500 μg of glycolipid per mouse. The similar level of granuloma forming activity was also demon-

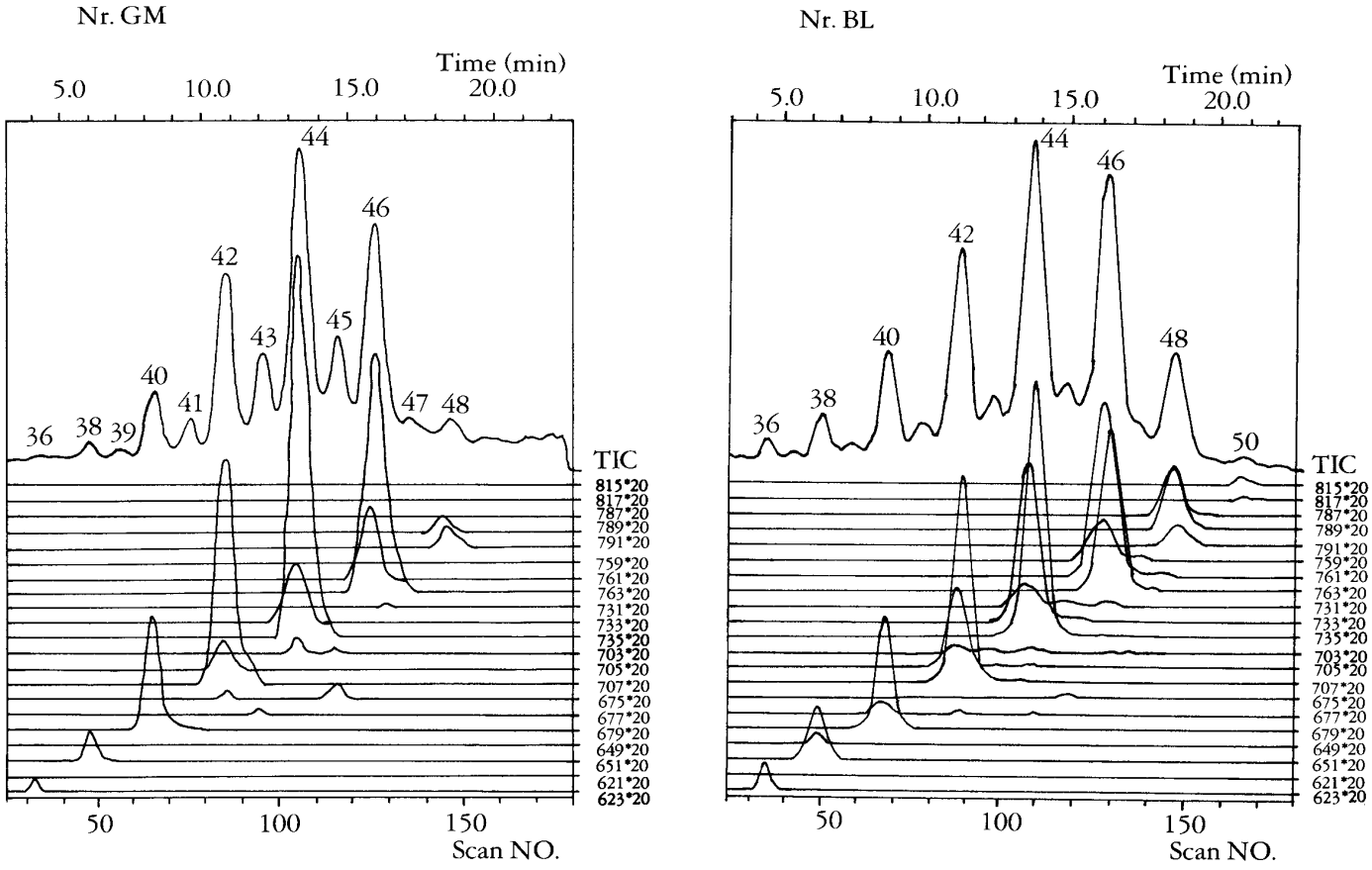


FIG. 7. *Mass Chromatograms of TMS Methyl Mycolates of the Glycolipid "GM" and Bound Lipid "BL" Fraction in N. rubra*

The figure indicated the mass chromatograms monitoring (M-15) ions of the individual TMS methyl mycolate species.

TABLE I. *Mycolic Acid Composition of Each Glycolipid Class*

Carbon No.	Trehalose monomycolate			Trehalose dimycolate			Glucosyl mycolate			Bound lipid (CWS)		
	Sat-	Mono-	Di-	Sat-	Mono-	Di-	Sat-	Mono-	Di-	Sat-	Mono-	Di-
36	tr			2.8	tr		tr	tr		1.8	tr	tr
37	tr			2.6	tr		tr	tr		0.4	tr	tr
38	1.5			4.3	0.4		1.8	tr		3.2	0.4	tr
39	tr	tr		2.0	0.4		0.7	tr		0.5	0.1	tr
40	4.5	tr		8.3	1.6	tr	8.0	tr		6.5	1.2	tr
41	3.0	tr		3.0	1.0	tr	3.1	0.2	tr	1.0	0.4	tr
42	12.9	3.3	tr	14.2	4.5	tr	18.8	1.1	tr	13.6	4.9	0.9
43	6.1	1.5	tr	2.2	1.0	0.4	4.6	0.7	tr	0.8	0.5	0.2
44	25.3	5.1	tr	17.8	7.9	1.0	29.7	4.6	tr	15.0	10.4	2.8
45	5.6	1.5	1.0	2.8	1.6	0.4	3.5	1.1	tr	0.5	0.8	0.2
46	17.2	4.8	2.3	6.9	3.9	1.0	13.5	5.1	tr	10.0	11.6	3.0
47	1.0	0.8	tr	1.0	0.8	0.4	0.4	0.2	tr	tr	0.2	0.2
48	1.3	1.0	tr	3.0	2.2	0.8	1.6	1.1	tr	1.4	4.4	3.9
49	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
50	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	0.2	0.4

Mycolic acid compositions are expressed as percent of the total.
tr: trace.

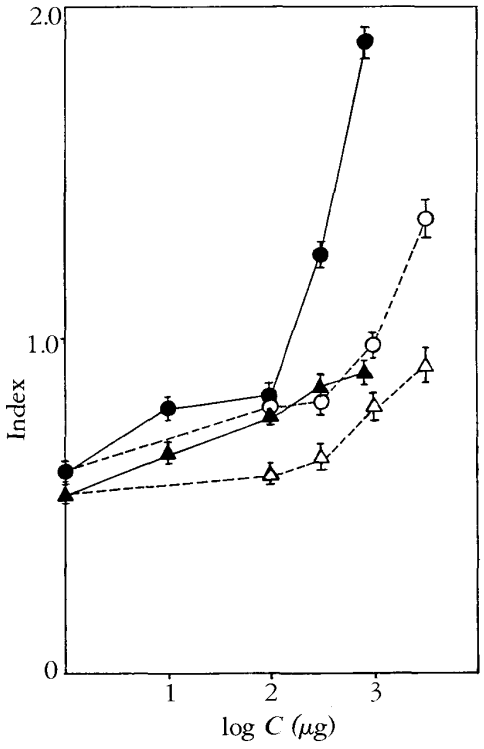


FIG. 8. *Lung Granuloma Formation in Mice by an i.v. Injection of Heat Killed Cells or the Total Extractable Lipids of N. rubra in W/O/W Micelles*
●, ○, *lung index,* ▲, △, *spleen index;* -----, *heat killed whole cells;* ——, *C-M extractable lipids.*

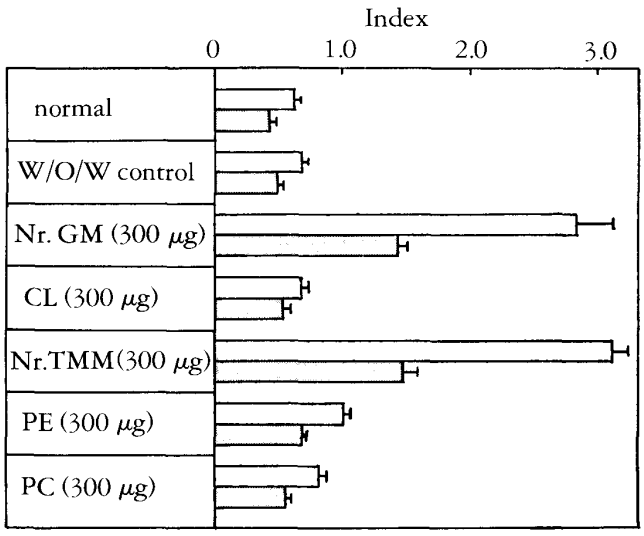
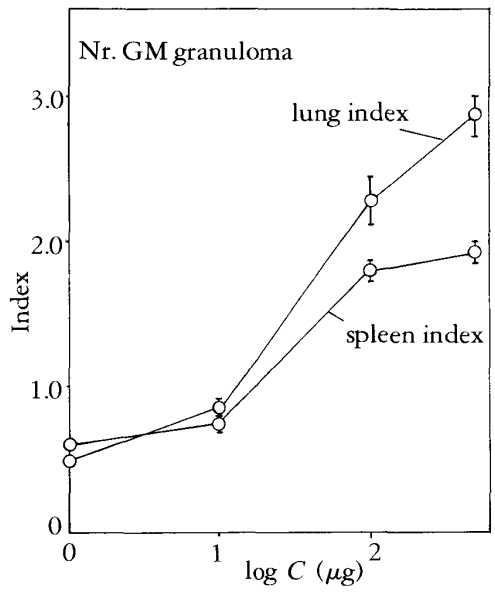


FIG. 9. *Lipid Specificity for Granuloma Formation in Lungs and Spleen in Mice by an i.v. Injection of the Separated Lipid in W/O/W Micelles*
□ *lung index;* ▨ *spleen index.*

strated in the glycolipids possessing different mycolic acid species obtained from other *Rhodococcus* or *Gordona* species and it was noted that these activities were comparable to those of synthetic adjuvant, 6-*O*-mycoloyl muramyl dipeptide (6-*O*-mycoloyl MDP).[4i,9]

Histological examination of granulomatous tissues one week after intravenous injection of the total or isolated glycolipid in W/O/W



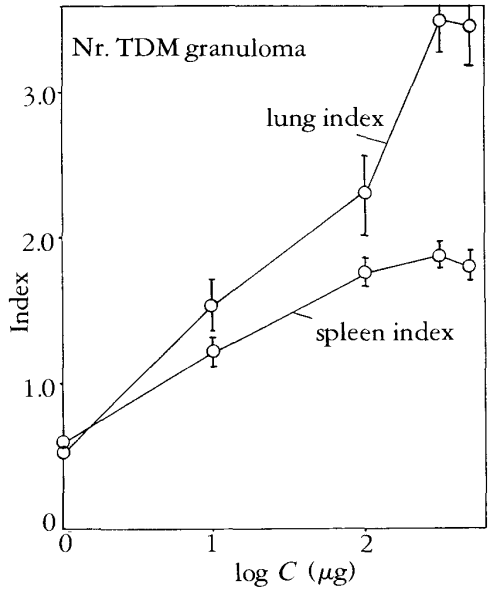


FIG. 10. *Dose Responses for Granuloma Formation in Lungs and Spleen in Mice by an i.v. Injection of Glycolipids "GM (Glucosyl Mycolate)" or "TDM (Trehalose Dimycolate)"*

showed a marked infiltration of a large number of monocytes or mature macrophages and a few immature epithelioid cells with polymorphonuclear neutrophiles around the oil droplets in lungs. In the case of spleen, one week after injection of glycolipids, granulomas were composed of closely packed monocytes and a few number of epithelioid cells. It was noted that a number of multinucleated giant cells were observed in the spleen one week after injection of glycolipid, although they are moderately large and seems to be foreign body type giant cells rather than Langhans giant cells. It was also noted that the highest activity for massive granuloma formation in lungs and spleen was observed with trehalose dimycolate (TDM) of *N. rubra* in which a tightly packed cellular infiltration appeared in lungs. A detailed histological or electronmicroscopica observation of the granuloma tissues after injection of C_{36-48} mycolic acids-containing glycolipids in mice has been described and published in a separate paper.[16c)]

The toxicity of mycolic acid-containing glycolipid was estimated by the decrease in the body weight of mice after intravenous injection of glycolipid micelles. Throughout these experiments, the decrease in the body weight was within 10—15 percent of the control animals and therefore, the toxicity of the glycolipids containing C_{36-48} mycolic acids appeared to be lower than those containing C_{70-80} mycolic acids in mycobacteria.

From the above results, it was concluded that glycolipids containing shorter chain mycolic acids ranging around C_{40-50}, corresponding to less acyl numbers such as trehalose monomycolate or monosaccharide such as glucose monomycolate, can also form granuloma in mice without protein antigens and therefore, seem likely to possess adjuvant activity.

DISCUSSION

Genera *Nocardia* and *Rhodococcus* are originally soil bacteria belonging to *Actinomycetales* and are taxa close to *Mycobacterium* phylogenically. The major species of *Nocardia* and *Rhodococcus* are non-pathogenic or opportunistically pathogenic for man, although the cell wall structures are basically identical to each other, possessing mycolic acids as their hydrophobic and arabinogalactan polysaccharide as hydrophilic components. Therefore, they are useful for the analysis of structure-biological activity relationships between host and parasite in mycobacterial infection. We have, to date, studied the structure and physiological functions of various mycolic acid molecular species which are the most characteristic component in cell walls of these types of bacteria.[2a,b,d,8,10)] "Cord factor", trehalose 6,6′-dimycolate, is a sole toxic glycolipid of tubercle bacillus and was first isolated and characterized by Bloch[11)] and Noll and Bloch.[12)] Later, however, the occurrence of similar glycolipids containing mycolic acids were reported widely in other organisms belonging to genera related to *Mycobacteria*.[13)] Therefore, it is difficult at this stage to elucidate the pathogenicity or toxicity of glycolipids in human type *Mycobacteria*. Furthermore, recent attention has been paid to the immunological properties of cord factor which possess adjuvant activities, macrophage activating activities and the enhancement of tumor immune response, of synthetic adjuvant muramyl dipeptide (MDP) or mycoloyl MDP and the cell wall skeletons of BCG or *N. rubra*.[4h,i,5,6,9)] To define the toxicity or the degree of contribution to the host-reaction of cord factors, it is essential to compare the structure of glycolipids in the light of both hydrophilic and hydrophobic moieties. Since only limited reports concerning the glycolipid structure and biological activities in *Nocardia* and related taxa have been published, we have commenced structural analysis using newer analytical techniques such as GC-MS and examination of the host reactive properties in various experimental animals. The present paper revealed that at least three classes of glycolipids possessing mycolic acids existed in the chloroform-methanol extracts: trehalose monomycolate, trehalose dimycolate and glucose mycolate. The latter compound has not been reported so far to occur in the genera. The mycolic acid compositions of each glycolipid were fully characterized by GC-MS analysis which also revealed that they coincided approximately to the total cellular mycolic acids reported previously.[2a,14)] However, it was also first demonstrated that the mycolic acid composition of each lipid class or lipid fraction differed significantly from each other and the specific lipid possessed a specific pattern of mycolic acid composition, even though the mechanism is not known. The most dramatic findings are the biological activities of these gly-

colipids. All three classes of glycolipids possessing mycolic acids in *N. rubra* showed a strong activity for granuloma formation in lungs and spleen in mice, even though the granuloma appeared to be non-specific (non-immunogenic) and of a foreign type, judging from the duration of time after injection and histological observation. Bekierkunst *et al.* first reported that mycobacterial cord factor produced epithelioid granuloma in mice[4c] and they also reported that a "semi-synthetic cord factor" was also granulomagenic. As Yarkoni *et al.* reported, a glycolipid structure may be essential for its granulomagenic activity.[3g] We expected initially that the granuloma inducing activity of *N. rubra* glycolipid would be low even if it did occur, since it has been reported that the toxicity of cord factors possessing shorter chain mycolic acids is lower.[15] On the contrary, the results were showed unexpected strong activity. At this stage, we cannot conclude what part of or to what extent the structure of cord factor and related glycolipids may contribute to the granulomagenic activity. However, by comparing the activities of glycolipids differing in mycolic acid moiety or in carbohydrate composition, we can define the most precise structure of natural glycolipids required for massive granuloma induction. We are also considering comparative studies for granuloma formation by glycolipids with or without protein antigen and examining the non-specific infection prevention activity or enhancement of antitumor activity by the granuloma thus produced. Similar comparative studies with rats or guinea pigs and BCG high responder or low responder mice are also now in progress. The aspects of the histological and electromicroscopic studies on granuloma formation in mice and other experimental animals are described in a following paper.

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